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14. ABSTRACT

The purpose of this concept award grant is to uncover potential drug targets for treatment of Neurofibromatosis type 2 (NF2). We planned a synthetic lethal screen, using RNAi technology to uncover protein kinases and phosphatases that are specifically required for the survival of NF2-null cells. We obtained and reformatted a murine siRNA library against all known protein kinases and phosphatases. We also obtained NF2flox/flox mouse embryo fibroblasts and used Cre recombinase to convert these to a NF2-/- genotype. We then tested a large number of transfection procedures to determine the most effective and least toxic method for delivery of siRNA into these cells, and optimized this procedure for a high throughput assay. Having carefully established the conditions for the screen, we are carrying out the main, most important experiment: the large scale screen for synthetic lethality.

15. SUBJECT TERMS
RNAi screen; synthetic lethality; kinome; phosphatome; NF2

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Jonathan Chernoff, M.D., Ph.D.

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INTRODUCTION:

The purpose of this concept award grant is to uncover potential drug targets for treatment of NF2. Specifically, we plan a synthetic lethal screen, using RNAi technology to uncover protein kinases and phosphatases that are specifically required for the survival of NF2-null cells.

BODY: We set ourselves four specific tasks. These were:

Task 1. Obtain and amplify mouse shRNA kinase library:

a. Prepare plasmid DNA from ~1000 kinase shRNA clones, using Qiagen kits.
b. Determine titer of adenoviruses by plaque method (Months 1-2).

Task 2. Convert plasmids to retroviruses:

a. Transfect Phoenix packaging cells with each member of the shRNA kinase library, using 96-well plates. Test efficacy of selected retroviral shRNAs against kinases thought to be germane to NF2 signaling, e.g., Pak1, Pak2, and MLK-3.

Task 3. Convert $Nf2^{flox/flox}$ MEFs to $Nf2^{-/-}$ genotype:

a. Infect $Nf2^{flox/flox}$ MEFs with a retrovirus expressing Cre recombinase and YFP, sort for YFP positive cells

Task 4. Screen shRNA kinase library for effects on growth and morphology of WT and NF2-null MEFs:

a. Measure density of growth in the presence or absence of transduced shRNAs.
b. Retest positives from Task 4a to confirm phenotypic effects.
c. Test for physical interaction of proteins targeted by shRNA and Merlin.

Progress

Tasks 1 and 2. There are several changes in design that are worth noting. First, we decided to use synthetic siRNA rather than plasmid-based shRNA. We did this because we found that the two main commercial shRNA libraries (the Hannon/Elledge Open Biosystems library and the Bernards NKI library) were difficult to work with due to issues of recombination and poor knock-down of several kinase genes tested. These libraries are rapidly evolving (*e.g.*, use of miRNA leader sequences to enhance knock-down, switch to Lentiviral backbone to increase range of infectability, *etc*.) but we were unwilling to wait for the needed technical improvements. In short, while these libraries, in their current incarnation, are useful in mass screens, they are less useful in well-by-well screens such as ours. We therefore obtained a murine kinome and phosphatome siRNA library from Qiagen. The human version of this library has been used by John Blenis to uncover kinases and phosphatases required for cell survival in HeLa cells (MacKeigan, Murphy & Blenis, NCB 7:591, 2005). For these reasons, we did not need to carry out the planned procedures initially listed as tasks 1 and 2, but instead had a new task: obtaining and reformatting the murine siRNA kinome and phosphatome library for use in our synthetic

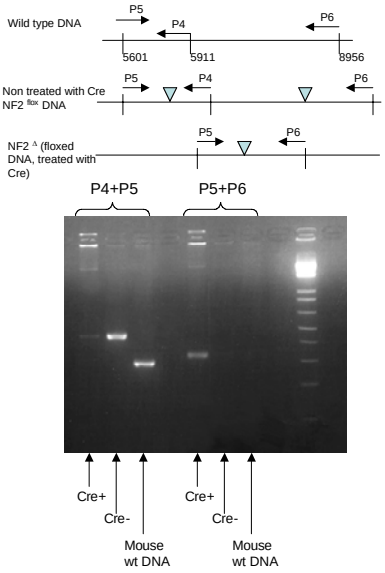

Fig. 1. The $NF2^{flox/flox}$ allele was detected, using primers P4 (5'-CTTCCCAGACAAGCAGGGTTC-3') and P5 (5'-GAAGGCAGCTTCCTTAAGTC-3'), yielding a 442-bp $NF2^{flox/flox}$ allele and 305-bp $NF2^{+/+}$ allele (when using DNA from WT MEF). The NF2Δ (mutant) allele was detected, using primers P5 and P6 (5'-CCTCTATTTGAGTG CGTGCCATG-3'), yielding a 338-bp NF2Δ (mutant) allele.

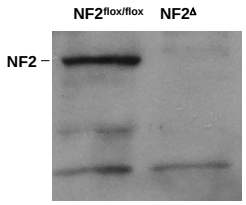

Fig. 2. Expression of NF2. Floxed NF2 cells were treated with Cre as described in Fig. 1. Cell lysates were probed with anti-NF2 sera.

lethal assay. Because the Qiagen library contains two duplexes for each gene, we made a version of the library in which these two duplexes are combined. This is similar to the strategy taken by the Blenis lab in their study of the kinome and phosphatome, and allows us to do half as many initial transfections.

Task 3. We obtained $NF2^{flox/flox}$ MEFs from Andi McClatchey (Harvard Medical School). These were left as is or converted to NF2Δ by infection with Adeno GFP (control) or Adeno Cre-GFP, respectively. Cells were sorted twice for GFP, then tested for genotype and phenotype as follows:

Genomic DNA was extracted from control and Cre-treated cells, and analyzed by PCR (Fig. 1). We data show that we efficiently converted the $NF2^{flox/flox}$ allele to NF2Δ, as demonstrated by the shift in size of PCR products. To test the effects of this conversion on NF2 expression, we carried out immunoblot analysis using NF2-specific antibodies. These results, shown in Fig. 2, demonstrate that removal of the floxed NF2 exons results in loss of NF2 protein. Finally, we also tested the growth properties of the control ($NF2^{flox/flox}$) and NF2Δ cells. Our results show that removal of NF2 profoundly alters cell proliferation, as expected for a tumor suppressor (Fig. 3).

Task 4. Given that we are using siRNAs, we needed to establish a successful transfection regimen to achieve effective mRNA knock-down. We tested the following lipid-based reagents: Lipofectamine 2000, Oligofectamine, RNAifect, Dharmafect, INTERFERin, Lullaby, HiPerfect, and Generaser. We found that the highest level of knock-down was achieved using Lipofectamine 2000 and HiPerfect, and that, in the latter case, efficiency was improved by incubating cells in the absence of serum during transfection. Using these conditions in cells in 6-well plates, we achieved

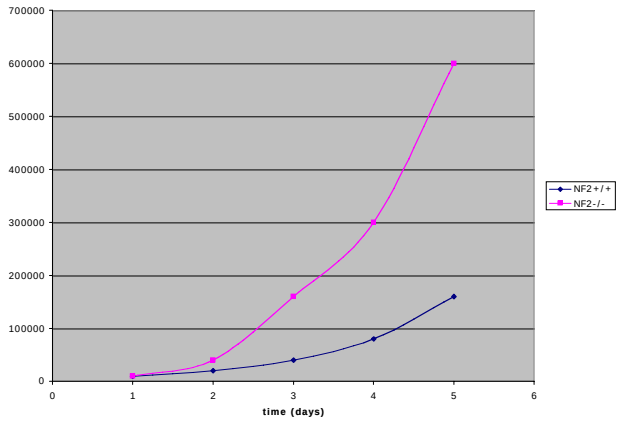

Fig. 3. Growth properties of NF2$^{flox/flox}$ *vs.* NF2Δ cells. 10,000 cells were plated per 35 mm, and the cell number was assessed every 24 h.

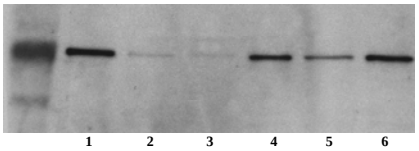

Fig. 4. Efficacy of siRNA. NF2Δ cells were transfected with Pak2 siRNA to final concentration of 10 nmol/l. An immunoblot for Pak2 was performed 48 hr post transfection. 1) OligoFectAmine; 2) LipoFectAmine2000; 3) HiPerFect (serum-free media); 4) HiPerFect (full media); 5) RNAiFect; 6) nontransfected cells.

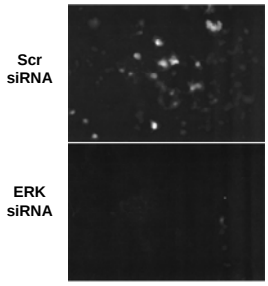

Fig. 5. Efficacy of siRNA in MEFs plated in 96-well format. NF2Δ MEFs were plated in a 96-well dish and transfected with scrambled or anti ERK siRNA, using HiPerfect. The cells were fixed and stained for ERK 48 hr post transfection.

KEY RESEARCH ACCOMPLISHMENTS: Bulleted list of key research accomplishments emanating from this research.

1. Obtained and reformatted murine kinome and phosphatome siRNA library.
2. Obtained NF2$^{flox/flox}$ MEFs, converted these to NF2Δ using Cre recombinase, and demonstrated that these cells lose NF2 expression and have increased cell proliferation.
3. Evaluated methods of siRNA transfection and optimized use of reagents.

REPORTABLE OUTCOMES:

There are not as yet any reportable outcomes.

CONCLUSION:

We have demonstrated the feasibility of the synthetic lethal screen, overcoming a number of logistical hurdles. As this project is not complete, we have therefore asked for a 1 year, no-cost extension. After a number of design changes and optimizations, the system is up and running, but the most important experiments lie in the months ahead.

REFERENCES:

None.

APPENDICES:

None.